ORAL MICROBIOME CHANGES ASSOCIATED WITH FIXED DENTAL PROSTHODONTIC RESTORATION

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ABSTRACT

Sarah Kay Youny Lee: Oral microbiome changes associated with fixed dental prosthodontic restoration
(Under the direction of Kimon Divaris)

The oral microbiome is a relatively unexplored component of oral health and disease. Common oral diseases, including dental caries and periodontitis, are now best understood as dysbiotic shifts of the oral microbial ecology. Prosthodontics is a cornerstone of clinical dentistry, serving to promote oral health through dental rehabilitation. However, it remains unknown if and to what degree prosthodontic treatment confers changes in the oral microbiome.

In this observational clinical study, we studied the effects of fixed dental prosthodontic restorative treatment on the oral microbiome composition. Prosthodontic patients' salivary samples were collected during their treatment course. Microbiome analyses relied upon whole genome sequencing (WGS) shotgun. Reads were aligned, mapped and analyzed to obtain measures relative abundance and diversity, as well as group and time differences. Results to-date have provided novel insights into oral microbiome changes during prosthodontic treatment that may characterize global transitions of clinical oral disease states to health.

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DNA	Deoxyribonucleic acid
EPR	Electronic patient record
FDP	Fixed dental prosthesis
HOMINGS	Human Oral Microbe Identification using Next Generation Sequencing)
MetPHlAn2	Metagenomic Phylogenic Analysis
mRNA	Messenger ribonucleic acid
MS	Mutans streptococci
NA	Nucleic acid
NGS	Next generation sequencing
NMDS	Non-metric Multidimensional Scaling
PC	Principal Component
PCoA	Principal Component Analysis
RPK	Reads per kilobase
STROBE	Reporting of Observation Studies in Epidemiology
rRNA	Ribosomal ribonucleic acid
tSNE	t-Distributed Stochastic Neighbor Embedding
WGS	Whole genome sequencing

INTRODUCTION

1. Oral microbial communities and their organization

The oral cavity is a dynamic environment built by communities of microbial cells consisting of bacteria, fungi, archaea, viruses, and protozoa (Wade 2013; Zaura et al. 2014). It is one of several interrelated systems of the body, including the gut, skin, and rectum, that make up the overall human microbiome and metagenome(Group et al. 2009; Human Microbiome Project 2012b). The cells of these microbiomes account for 90% of cells of the human body (Savage 1977; Wilson 2008). To explore this crucial capacity, relatively recent and extensive investigations have been done to better understand the oral microbiome organization of the oral cavity and its many parts in health and disease.

1.1. Defining the oral microbiome

The oral cavity is an ecosystem containing various habitats that consist of communities of commensal, symbiotic, and pathogenic microorganisms (Lederberg and McCray 2001). These communities are referred to as microbiomes (Ursell et al. 2012).

Seven major phyla of bacteria dominate the oral microbiome: *Firmicutes, Proteobacteria Actinobacteria, Bacteroidetes, candidate division TM7, Spirochaetes*, and *Fusobacteria* (Dewhirst et al. 2010; Lazarevic et al. 2010; Wade 2013). These species have been found to vary within site-specific communities of the oral cavity - specifically supragingival dental plaque, saliva, and mucosa (Xu et al. 2015). The sites differ in their species composition and relative abundance (Dewhirst et al. 2010; Xu et al. 2015; Zarco et al. 2012; Zaura et al. 2014). Further,

significant variations are also observed with age and the stage of dentition, suggesting that the composition of the oral microbiome has a temporal component (Xu et al. 2015).

The oral microbiome consists of a core set and variable set of microorganisms that are in a flux of symbiosis and dysbiosis based on innate and acquired host factors such as genetics and oral hygiene habits. The core microbiome is a patterned diversity displayed in clinically healthy oral environments and is consistently shared between different individuals. On the other hand, the variable microbiome consists of microbial communities unique to the individual (Bik et al. 2010; Zarco et al. 2012; Zaura et al. 2014).

1.2. Biofilms and the oral microbiome

The oral bacteria are predominantly organized in biofilms within the oral cavity (Marsh 2006; Socransky and Haffajee 2005). The physical integrity of the biofilm is based upon an exopolymer matrix structure that encapsulates a specific compositional and spatial design of microorganisms (Jenkinson and Lamont 2005; Sbordone and Bortolaia 2003). The microbial composition develops by way of inter-species interactions as well as interactions with the host environment and its subsequent reaction to the inhabiting microorganisms that leads to a process of succession (Bick et al. ; Sbordone and Bortolaia 2003; Socransky and Haffajee 2005). Quorum sensing, a bacterial mode of communication in which quorum sensing molecules are produced and detected by cells, has been suggested as one of the primary mechanisms by which a biofilm regulates the balance of healthy and pathogenic concentrations of microbial species (Wade 2010).

Evidence shows that the relative abundance of bacteria within the oral microbiome is based on ecological location within the oral cavity and time - from the aspects of the maturation of a niche's state of health or disease and age of the human host (Costalonga and Herzberg

2014). These biotas are essentially biofilms that organize both spatially and temporally(Sbordone and Bortolaia 2003).

1.3. Saliva and the oral microbiome

Saliva is a biological fluid made of water, proteins, and inorganic and organic substances contained within the oral cavity. While the oral microbiome has specific ecological organization, the salivary microbiome has been shown to be representative of the overall oral microbiome due to its ability in collecting the shedding surfaces of these oral niches(Fabian et al. 2008; Yamashita and Takeshita 2017). It can serve as a global marker of the oral microbiome because of its encompassing nature in which saliva directly contacts and carries components derived from other oral structures such as gingival crevicular fluid, cell debris, plaque, nasal and bronchial secretions, lining cells, blood, exogenous substances, and bacteria(Curtis et al. 2011; Fabian et al. 2008; Fox 1989; Kaczor-Urbanowicz et al. 2017; Kaufman and Lamster 2000; 2002; Lee 2009; Liu and Duan 2012; Mandel and Wotman 1976; Saliva: Its role in health and disease. Working group 10 of the commission on oral health, research and epidemiology (core) 1992). It contains mRNAs, proteins, and DNAs from local tissue as well as tissues from distant sites.

Even though bacterial species tend to be site-specific (Aas et al. 2005; Dewhirst et al. 2010; Paster et al. 2006; Wade et al. 2005; Xu et al. 2015), previous evidence suggests that saliva houses bacteria, both aerobic and anaerobic, that characterize the state of other oral niches such as supragingival plaque and subgingival, periodontal pockets(Asikainen et al. 1991; Bowden 1997; de Jong et al. 1984; Greenstein and Lamster 1997; Simon-Soro et al. 2013; Umeda et al. 1998). Saliva does disproportionally consist of microbial cells deriving from the tongue's biofilm, but it serves as a reservoir for shedding surfaces found in the oral cavity, usually from desquamating epithelial surfaces (Costalonga and Herzberg 2014; Simon-Soro et al. 2013).

Saliva has been shown to exhibit long-term temporal stability (Belstrom et al. 2016b; Rasiah et al. 2005; Stahringer et al. 2012; Yamanaka et al. 2012; Zhou et al. 2013) and affected by the host and host's systemic condition (Lira-Junior et al. 2018; Zaura et al. 2017), but its composition has been found to be altered based on external factors such as use of systemic antibiotics, as well as chemo- and radiotherapies (Lazarevic et al. 2013; Xu et al. 2013). However, the salivary bacterial profile variance has been found to be comparable, at around 13.5%, to variations found in distributions of genera in neutral genetic markers between individuals in human populations(Nasidze et al. 2009). This implies that saliva may be applicable in a global sense in which geographic location may not necessarily impart a distinct composition.

As such, saliva can be used as a tool for assessment and diagnosis, as well as potential predictive indicator of oral microbial health status (Belstrøm 2016; Belstrom et al. 2016a; Kaczor-Urbanowicz et al. 2017; Kaufman and Lamster 2000; 2002; Lee 2009; Liu and Duan 2012).

1.4. Next generation sequencing (NGS)

The process of characterizing the oral microbiome consists of a conglomeration of techniques. Traditional cultured isolates of bacteria have yielded approximately 280 identifiable species(Dewhirst et al. 2010). However, the majority of oral bacteria cannot be cultivated and thus are unobservable unless cultivation-independent molecular methods are used (Wu et al. 2014). An early high-throughput technology that was based upon 16S ribosomal RNA gene sequencing was made possible because this microbial gene sequence is found in prokaryotes and demonstrates high variability that can be utilized in the identification of a broad spectrum of microbes(Ahn et al. 2011; Dewhirst et al. 2010). 16S rRNA pyrosequencing, Illumina

sequencing and the Human Oral Microbe Identification Microarray (HOMIM) have been used by several studies and were shown to be valid and efficient means of studying the oral microbiome(Ahn et al. 2011).The core data of the oral microbiome has been accumulated to form the Human Oral Microbiome Database, a collection of microbial reference genomes consisting of known species, unnamed isolates, and unnamed and uncultured phylotypes recognized by the 16S rRNA sequence information (Ahn et al. 2011; Dewhirst et al. 2010). This database organizes the defined human oral microbial taxa in a standardized manner that is accessible for research and dissemination(Ahn et al. 2011). It is also part of a greater conglomeration known as the Human Microbiome Project in which bacterial genome sequences are being collected in major ecological sites of the body – the nasal cavity, oral cavity, gastrointestinal tract, and urogenital tract (Ahn et al. 2011; Dewhirst et al. 2010; Morgan et al. 2013).

1.4.1. Whole genome sequencing (WGS) shotgun - metagenomics

WGS, a type of NGS approach, sequences large quantities of DNA, ordering the nucleotides such that variations can be identified in any part of the genome (Bick et al. ; Genomic research 2018; Human Microbiome Project 2012a; Ng and Kirkness 2010; Weinstock 2012). It taxonomically profiles a microbial community to the species, and more detailed, the strain level (Weinstock 2012). WGS also enables functional profiling of metagenomic and metatranscriptomic sequence data, including aggregated whole-community level pathway reconstruction (Abubucker et al. 2012; Fodor et al. 2012; Lozupone et al. 2006; Nyvad et al. 2013; Schloss et al. 2009; Segata et al. 2013; Weinstock 2012).

WGS can be employed using two methods: a reference-based assembly and de novo assembly (Ng and Kirkness 2010). With the reference-based assembly, short lengths of DNA

sequence (reads) are compared to an existing reference genome sequence such that a consensus sequence can be formed (Ng and Kirkness 2010). With de novo assembly, the total microbial DNA is directly sequenced through overlapping of comparable sequence reads that are overlapped to form long sequences (contigs) (Ng and Kirkness 2010; Nyvad et al. 2013; Weinstock 2012). The process of generation, assembly, organization and analysis of WGS data is complex, computationally, time and resource intensive, but can be highly informative (Bick et al. ; Weinstock 2012).

By utilizing WGS, a more comprehensive compositional view of microbial communities can be generated.

2. Oral health and disease

The oral microbiome is a highly individualized, dynamic composition of commensal microbial species with development and interactions influenced from host genetic and environmental factors (Costalonga and Herzberg 2014; Gomez et al. 2017; Wade 2010).

2.1. Previous models of oral disease and bacteria

The mechanisms of oral diseases such as caries and periodontitis have historically been understood as infectious microbial processes, in which disease-specific bacteria induce a negative impact on susceptible oral structures. This pathogenicity-driven model related disease occurrence to certain microorganisms that had the ability to overcome and/or surpass host defenses.

2.1.1. Caries

The caries process was traditionally viewed as a bacteria-specific disease. *Mutans streptococci* (MS) and *Lactobacillus acidophilus* were identified as the key bacteria associated with caries (Hamada and Slade 1980; Liljemark and Bloomquist 1996; Loesche 1986; van Houte

et al. 1994). Theories that attempted to explain dental decay emerged and were organized into specific versus non-specific plaque hypotheses (Kleinberg 2002; Loesche 1986).

Caries-pathogen acquisition was hypothesized to occur within a "window of infectivity", in which colonization of MS occurs at the time of primary teeth eruption(Caufield et al. 1993). Furthermore, previous research then linked the source of vertical transmission from mother, or other closest caregiver, to child, describing infants with MS also had mothers and caregivers carrying this pathologic bacteria(Berkowitz 2006; Berkowitz et al. 1975). Horizontal transmission of MS, in which microorganisms are transferred between members of a group, had also been found to be plausible as children sharing a daycare setting and non-mother members in the immediate family have been found to share genotypes of MS(Mattos-Graner et al. 2001; van Loveren et al. 2000).

2.1.2. Periodontal disease

Periodontal diseases, ranging from gingivitis to aggressive periodontitis, were investigated based on species-association to a particular type of disease (Moore et al. 1983; Moore et al. 1982; Paul 1970; Slots 1979; Socransky Sigmund and Haffajee Anne 1994; Socransky and Haffajee 1992; Tanner et al. 1979). Microbes distinctive to a diseased state were identified as keystone pathogens and included *Porphyromonas gingivalis, Tannerella forsythia* (previously known as *Bacteroides forsythus*), and *Actinobacillus actinomycetemcomitans* (Moore and Moore Lillian 1994; Proceedings of the 1996 world workshop in periodontics. Lansdowne, virginia, july 13-17, 1996 1996; Socransky Sigmund and Haffajee Anne 1994). The concept of pathogenic bacterial complexes, particularly gram-negative varieties, then emerged, based on studies of microbial profiles derived from plaque samples that were analyzed via DNA probes in checkerboard hybridization assays, in which particular species were commonly found together

(Socransky Sigmund and Haffajee Anne 1994; Socransky and Haffajee 2005). The complexes were organized by a color-code system that indicated the microbial clustering, community ordination, and disease states as complexes and their pathogenic nature changed based on disease progression (Socransky and Haffajee 2005). The occurrence of periodontal disease was based on: 1) virulence of a bacterial pathogen, 2) the local environment, and 3) host susceptibility (Haffajee and Socransky 1994).

2.2. Current understanding of the oral microbiomes in health and disease

It is now understood that the processes of health and disease, as well as their dynamic balance, are community-driven, rather than pathogen-specific(Adler et al. 2013; Cephas et al. 2011; Ling et al. 2010; Sbordone and Bortolaia 2003; Xu et al. 2015). Microbial interactions, over a period of time and with influence from both physiologic host development and environmental factors such as dietary habits and hygiene, cause changes in the abundance and ergo, metabolism and expression of microorganisms within the oral environment (Bernard et al. 2012; Costello et al. 2012; Sbordone and Bortolaia 2003). The resulting host-bacteria responses are then indicative of whether health is preserved or disease is elicited (Buskermolen et al. 2018; Cosseau et al. 2008; Costello et al. 2012; Krisanaprakornkit et al. 2000; Peyyala et al. 2012).

Microbial biofilm shifts contribute to oral diseases (Dewhirst et al. 2010; Scannapieco 2013; Zarco et al. 2012). Biofilms can be viewed as a subset of a microbiome and demonstrate specific microorganism organization, communication, and functions(Faveri et al. 2015; Hajishengallis and Lamont 2012; Lin 2017; Marsh 2004; 2006; Sbordone and Bortolaia 2003; Teles et al. 2012; Wang et al. 2014). More than 65% of bacterial infections are biofilm-related, and it is the multiple organisms within a biofilm that can maintain health or cause disease (Lewis 2001). Common oral diseases such as caries and periodontitis are not simply pathogen-driven

conditions but rather clinical manifestations of dysbiosis occurring in the supragingival and subgingival oral microbiome, respectively (Jorth et al. 2014). In a state of symbiosis, the core and variable microbiomes interplay with their host and the host's factors to promote and maintain health. When disrupted, dysbiosis occurs and thereby, the disease process proceeds.

2.2.1. Caries

Investigations to understand the differences presented in a microbiome of a caries-free versus caries-active person demonstrate the complexity of bacterial diversity and interactions within a temporal scheme. It is understood that in infancy, heritable bacteria within the oral microbiome have been found to not be associated with caries pathogenicity, but with time, have reduced abundance (Gomez et al. 2017). The biofilms associated with a homeostatic state of health become disrupted, causing an ecological change in which microbial species tolerating the unbalanced state, become more dominant, and by which, a compositional change is incurred ((Marsh 2006; Nyvad et al. 2013; Takahashi and Nyvad 2011). Whereas, environmentally derived bacteria are more prevalent with age and more likely to cause caries (Gomez et al. 2017).

Novel species and their relative abundance in non-carious and carious states have been further identified through culture-independent methods (Belstrom et al. 2017b; Yang et al. 2014; Yang et al. 2012). Even though a large abundance of MS and *L. acidophilus* has continued to correspond to the disease-associated microbiota, these species are also notably present in non-diseased oral cavities or are absent in diseased states, suggesting that either these bacteria are part of the commensal environment of the microbiomes in the oral cavity, the proportion of the species and its functions may promote disease rather than presence alone being indicative of disease, or that a more specific strain of the species may contribute to the dysbiosis (Belstrom et al. 2017a; Belstrom et al. 2014; Belstrom et al. 2015; Belstrom et al. 2017b; Costalonga and

Herzberg 2014; Eriksson et al. 2017; Simon-Soro et al. 2018). Taxa likely associated with caries are impacted by environmental influences and show variation based on sucrose consumption and age (Costalonga and Herzberg 2014; Gomez et al. 2017). Bacterial functions can also be modified on the basis of the ecological changes that affect the environment, enabling different niches to form as the microorganisms adapt (Duran-Pinedo and Frias-Lopez 2015). Health-associated taxa change over time and interestingly show less abundance with increased sucrose intake (Gomez et al. 2017).

2.2.2. Periodontal disease

The distinctive presence and greater concentration of known disease-associated bacteria in periodontitis has been established. Further, a pattern of previously uncultured, but disease-associated microbes has been identified. But, the process by which this particular community shift occurs and establishes itself remains unclear.

It has been suggested that periodontitis operates in an initiator-promoter like causal mechanism where the microorganism composition sets up the disease process and the host's response facilitates disease expression (Teles et al. 2013). Periodontitis-associated biofilms not only have their own make-up of microbes, but also a distinctive metabolism and function that promotes virulence (Teles et al. 2013). In conjunction to this, the host immune-inflammatory response is triggered, which sets forth the destruction of tissue.

Viruses, including Epstein-Barra virus-1, human cytomegalovirus, and herpesviruses, have been detected in periodontal diseases (Saygun et al. 2008; Slots and Contreras 2001; Sugano et al. 2004). The viral etiology of disease, in the context of synergistic viral-bacterial infection, may exacerbate bacterial pathogenicity through enhancement of the virulence or depression the clearance of bacteria (Barton et al. 2007; Slots 2010). Viruses also have the

capacity to impair to immune or modulate immune response to periodontal disease (Lin et al. 2008; Lin and Li 2009; Loenen et al. 2001; Mogensen and Paludan 2001; Slots 2009; Slots et al. 2006).

2.3. Oral health and general health

The oral cavity is one of the key gateways of generalized health (Dewhirst et al. 2010; Oral health in america : A report of the surgeon general 2000). The oral cavity's condition determines and reflects the state of an individual's physiological, psychological, and social status of health (Antonoff 1975; Oral health in america : A report of the surgeon general 2000). Systemic diseases like diabetes, heart disease, and respiratory diseases have been found to be associated with poor oral health status and can both affect and be affected by oral health. Individuals displaying immune-incompetency are particularly susceptible to development of serious, systemically-involved complications from oral sources of infection (Oral health in america : A report of the surgeon general 2000).

3. Prosthodontics and oral health

Restoration of oral structures is a functional and esthetic necessity by way of addition of anatomy that is not present due to congenital or acquired means. These restorations require the integration of prosthetic, or artificial, components. Dental providers engage in this form of treatment, be it from direct restorations, such as direct and indirect tooth restorations like amalgam or composite resin fillings and crowns, or, on a larger scale, reconstruction of the jaw after trauma or disease.

3.1. Defining dental prosthetic reconstruction

For the restoration of dentitions, utilized prosthodontic restorations can reconstruct a single tooth or multiple teeth, and can amount to the entire mouth. The treatment goal is to promote structural integrity that is supportive of function, via mastication and phonetics, as well as socio-psychological interactions related to one's appearance and the capability of completing functional tasks in a socially acceptable, unimpeded manner (Franks 1976; Rosenstiel et al. 2016).

3.1.1. Fixed dental prosthesis (FDP)

The fixed dental prosthesis (FDP) is a restoration that is secured to a natural tooth or dental implant abutment(The glossary of prosthodontic terms 2005). A tooth-borne FDP, commonly referred to as a crown, encompasses the majority of a tooth in order to recreate the appearance and functions of the tooth it is restoring. The FDP can also replace missing dentition in its bridge form, by which two teeth, covered by a single FDP, abut and are connected to a single-tooth prosthesis (The glossary of prosthodontic terms 2005).

3.2. Indications for dental reconstruction

The need for treatment through dental prostheses may arise from compromised oral health. Edentulism, the state of missing dentition, continues to be a significant health issue (Cooper 2009; Felton 2009). It mostly occurs as a consequence of the most common oral diseases (caries and periodontal disease), which can lead to tooth morbidity and tooth loss if left untreated. Dental trauma and cancers of the head and neck area are additional, yet less common indications for dental reconstruction.

The concept of the prosthetic rehabilitation emerged from notion that restoration of an individual's occlusion would improve the state of the supporting structures and thereby, oral

health(Franks 1976; Goldman 1949; McLean 1978; Yanase and Le 2014). Occlusion is restored through such that normal and healthy function can occur. With improved function, it was observed that blood circulation is improved(Goldman 1949).

3.3. Prosthodontic treatment impact on general health

Studies have evaluated the outcomes of prosthodontic treatment on quality of life and general health. In general, patients who had undergone prosthetic treatment of any kind, including removable and fixed prostheses, reported a self-perceived improvement in their general health (Reissmann et al. 2011; Wickert et al. 2014).

3.4. Biological considerations for tooth-borne FDP restorations

Treatment with FDP involves diagnosis, planning, treatment, and maintenance. These steps in the process are aimed towards establishment of reconstruction of the dentition within a stable state of oral health, free of disease.

The process of restoration requires the creation of a sound and intact tooth foundation. This necessitates the removal and management of disease (Rosenstiel et al. 2016; Zitzmann et al. 2010). Though it has been reported that FDP restorations have an average lifespan of 10 years, complications that compromise the restoration or cause failure are anticipated (Tan et al. 2004).

Caries, pulpal injury, and periodontal disease are the leading causes of biological complications in tooth-borne fixed prosthetic treatment(Goodacre et al. 2003; Pjetursson et al. 2007; Pjetursson and Lang 2008; Tan et al. 2004; Yanase and Le 2014). This suggests that the longevity of a fixed prosthetic restoration is dependent on appropriate therapy of biologic factors that are attributable to and/or contribute to these complications. Placement of exogenous items in the oral cavity, (e.g., restorative materials and cements), likely impact the host its microbial

ecology. In return, these interactions can also affect the status of the prostheses and their long-term success.

While technical execution and fabrication of a quality prosthesis are important in the more immediate success and survival of a restoration and its tooth component, a favorable long term prognosis relies on the management of factors surrounding and outside of the parameters of the restoration (Antonoff 1975; Valderhaug 1980; Yanase and Le 2014).

4. Statement of Purpose

Prosthodontic dental treatment is the cornerstone of dental practice as it relates to the care and rehabilitation of a compromised dentition, usually due to the results of common oral diseases. However, it remains unknown if and to what degree prosthodontic treatment confers any (positive) changes in the oral microbiome. This study seeks to address this knowledge gap via the following aims:

Aim 1. Evaluate the salivary oral microbiome composition and diversity using next-generation sequencing (NGS) methods, arising from various clinical states of oral health (e.g., caries, periodontal disease, diabetes mellitus) as assessed in a cross-sectional manner.

Aim 2. Determine the effects of fixed dental prosthodontic restorative treatment on the salivary oral microbiome composition and diversity, in a prospective manner.

MATERIALS AND METHODS

1. Study design

This study was approved by the Institutional Review Board at UNC (UNC IRB #16-0040) and followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines on reporting observational human research studies. Participants were recruited from the UNC Graduate Prosthodontics clinic in Chapel Hill, NC between November 2016 and February 2018 and all provided written informed consent**.**

The study was designed as an observational, prospective cohort study investigating changes in the oral microbiome associated with prosthodontic dental rehabilitation. Unstimulated, whole saliva samples were obtained before, during, and after the completion of prosthodontic treatment. The participants are asked to provide three salivary samples at pre-defined time points within their treatment scope (pre-treatment, during, and post-treatment) using the OMNIgene ORAL OM-501 (DNA Genotek, OraSure Technologies, Canada) saliva collection kit.

2. Patient selection

Eligible study participants are UNC School of Dentistry patients who have been planned for, agreed to, and committed to undergo prosthodontics treatment with a graduate prosthodontic resident provider at the Graduate Prosthodontics clinic. The sample reported here comprises 17 adult patients between the ages of 40-78 years old requiring at least six fixed dental prosthetic restorations. Medical and dental histories were obtained at the time of screening and initial sample collection.

2.1 Inclusion criteria

Eligible participants must have met the following inclusion criteria: adult males or females who are years or older, able and willing to follow study procedures and instructions, able to read, understand and sign the consent form, in good general health, agree to undergo prosthodontic treatment consisting of, at minimum, definitive placement of six (6) units of any type of fixed dental prostheses (full coverage crowns or retainer crowns, pontics, inlays, onlays, implants).

2.2. Exclusion Criteria

Individuals are not be eligible for study participation if any of the following exclusion criteria apply: had a chronic disease with oral manifestations, exhibited gross oral pathology, received treatment with antibiotics for any medical or dental condition within 6 months prior to the screening examination, exhibited active periodontal disease, received chronic treatment (i.e., two weeks or more) with any medication known to affect periodontal status (e.g., phenytoin, calcium antagonists, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs, aspirin) within 6 months of the screening examination, received ongoing medications initiated less than 6 months prior to enrollment (i.e., medications for chronic medical conditions must be initiated at least 6 months prior to enrollment).

2.2. Continuance criterion

If in the duration of the study, any medical and/or dental status changes occur and apply to the exclusion criteria, the subject will be withdrawn.

3. Data collection and analysis

3.1. Electronic patient record (EPR) data

Dental and medical records are accessed via the school of dentistry EPR system to confirm that participants fit within the study's inclusion criteria and to also derive demographic, medical history and dental treatment information for eligible participants. Each participant's initial/presenting oral condition (e.g., clinical charting), based on the graduate provider's clinical examination and the planned restorative reconstruction, is recorded.

3.2. Clinical procedures

The first salivary sample (pre-treatment salivary sample) is obtained at any visit prior to the initiation of fixed dental prosthetic treatment from the patient's presenting restorative condition. The second sample is obtained at any appointment in which active oral disease have been managed but before placement of any definitive fixed dental prosthetic restorations (e.g., time of final impression). The third salivary sample is planned to be obtained one to two months after completion of the restorative reconstruction, during a post-operative follow-up examination treatment.

4. Saliva sample collection

The OMNIgene ORAL OM-501 (DNA Genotek, OraSure Technologies, Canada) saliva collection kit is used for all sample collections. The participants are instructed and verbally confirmed to not have had any solid or liquid oral intake for at least 30 minutes prior to saliva collection. Following the manufacturer's instruction, unstimulated, whole saliva is collected from the patient via expectoration, under supervision of a trained study assistant for as long as it is necessary to collect the required amount (2mL) of saliva.

5. Sample preparation, nucleic acid extraction, WGS shotgun

After total nucleic acid extraction from the saliva samples according to the manufacturers instructions, 1 ng of intact genomic DNA was processed using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA). Next, tagmented DNA was amplified via a limited-cycle PCR programme adding index 1(i7) and index 2(i5) (Illumina) in unique combination for each sample, as well as primer sequences required for cluster formation. Each library was purified using Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA) and quantified with Quant-iT™ PicoGreen® dsDNA Reagent (Molecular Probes, Thermo Fisher Scientific division, Waltham, MA). The resulting pool was heat denatured before loading on the HiSeq reagent cartridge and on the HiSeq 2500 instrument (Illumina). 150 base-pair paired-ended sequencing was carried out using a HiSeq SBS V4 (250 cycles) sequencing kit according to manufacturer´s instructions in the presence of 10% PhiX control (Divaris et al. In Press).

6. Bioinformatics pipeline and statistical analyses

Pair-ended **s**equencing reads were de-multiplexed and converted to FASTQ. Quality was assessed with FastQC. Reads were then aligned, human reads were removed, and all reads were converted to a single single-read dataset. Microbial community profiling was done using MetaPhlAn2.2. Further analyses were based on measures of relative abundance and diversity, measures relative abundance and diversity, selected species identification, as well as group and time differences in these metrics, using parametric (e.g., *t* test), non-parametric (e.g., Wald X^2) tests and graphical means. These included principal component analyses, PCoA, Scree, NMDS and tSNE plots. Due to the exploratory nature and pilot character of the study, corrections for multiple testing were not applied (Divaris et al. In Press).

RESULTS

1. General data

At the 1 year and 3 months since initiation of this investigation, 21 salivary samples were obtained from 17 subjects, with 4 subjects having had samples from 2 visits. Baseline demographic data and clinical characteristics of the subjects are summarized in Table 1.

Table 1. Demographic and clinical data

Variable	No	Yes	NA	Total
Caries	6	11	-	17
Perio	8	9	-	17
Gingivitis	4	13	-	17
Diabetes	5	12	-	17
second visit	13	4	-	17
Gender	Female: 4, Male: 13			17
Smoker	0: 3, 1: 12, 2: 1, NA: 1			17
Implant	A: 8, B: 1, AB: 5, NA: 3			17
Age	Min 40, Med 67, Max 78			17

A: Implant placement during treatment, B: Implant placement prior to treatment, AB: Implant placement prior to treatment and during treatment, NA: No implant placement

At baseline, samples were grouped based on caries status (absent versus present) for comparisons and distribution analyses.

The average abundance of genes and pathways per sample, as normalized for sequencing depth and gene length, was assessed in RPK (Reads Per Kilobase) and presented in Figure 1. The gene abundance distribution of samples and path abundance distribution of samples were further transformed by taking the logarithm to the base of 2 (figure 2, figure 3).

Figure 1. The average abundance of gene (in RPK) and pathways per sample at baseline

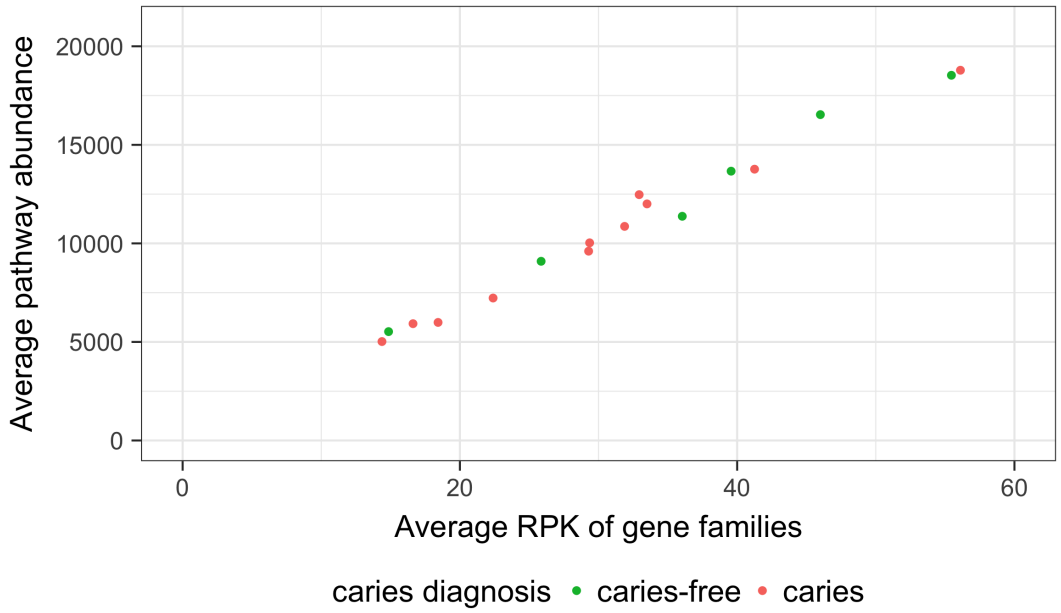
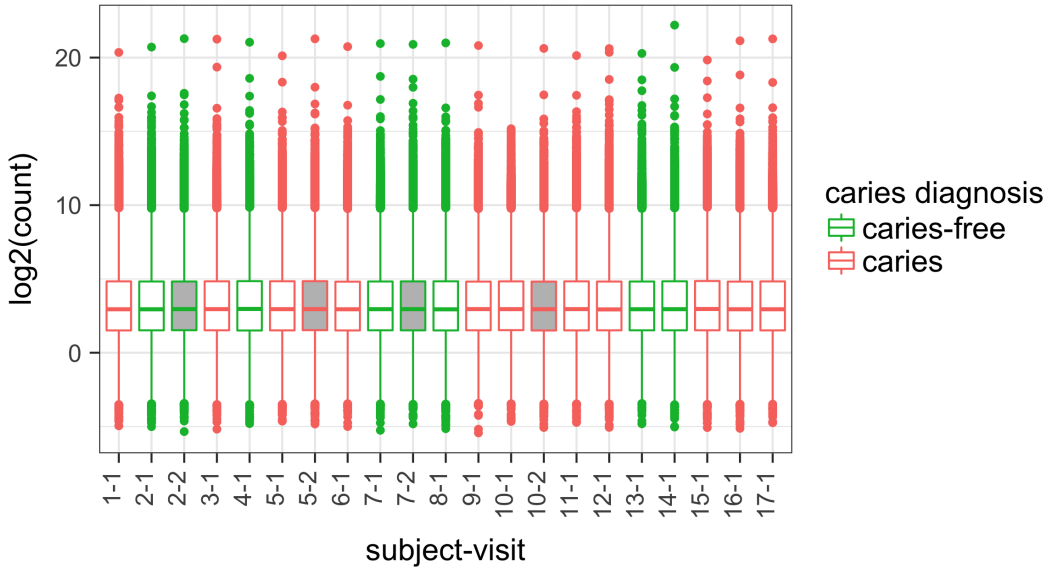
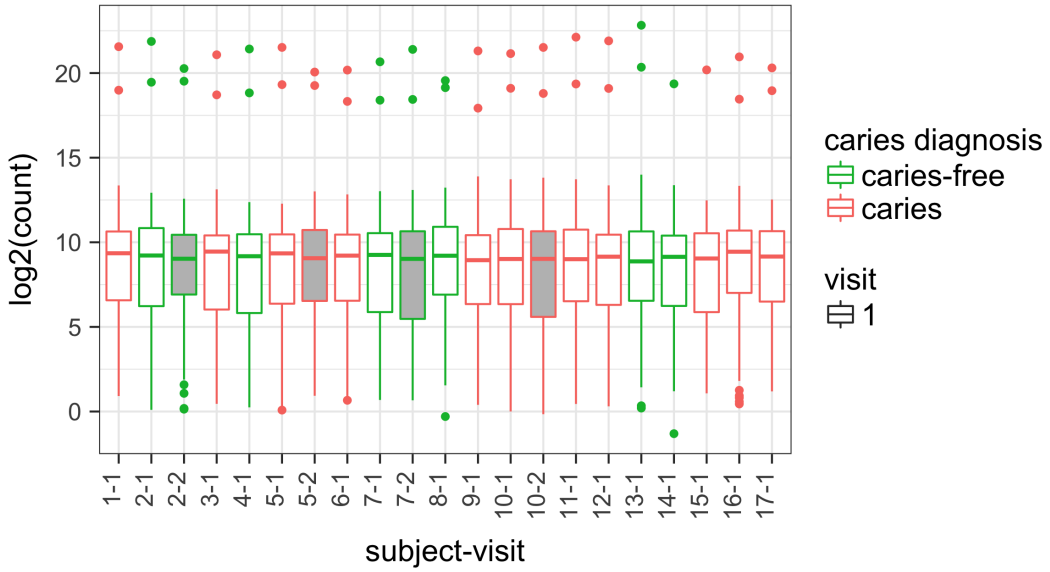


Figure 2. Gene abundance distribution of each sample (in RPK)



Zero counts excluded, Gray boxes represent 2nd visit

Figure 3. Pathway abundance distribution of each sample



Zero counts excluded, Gray boxes represent 2nd visit

No subject demonstrated an excessively small or large mean of RPK (genes) or mean path abundance. The abundance also did not demonstrate association with dental caries diagnosis.

2. Salivary metagenome analysis: Species-level

Metagenomic Phylogenic Analysis (MetaPhlAn2), a Maker Gene Based Taxonomic Assignment strategy was applied for identification of the microbial taxa from the salivary metagenome. The number of species, alpha, and beta diversities were studied(Whittaker 1972). For testing group mean differences, two-sample t-tests, or more generally Wald tests, were conducted based on linear models with the continuous covariates. Because this is an exploratory study, p-values were not routinely corrected for the multiple testing.

To compare the similarity of composition of bacteria between samples, the dimension of the compositional data was reduced and possible clustering was assessed. For dimension reduction, the species were screened by detection rate of 0.002 and prevalence threshold of 0.5, and methods including Non-metric Multidimensional Scaling (NMDS) (Kruskal and Wish 1978), t-

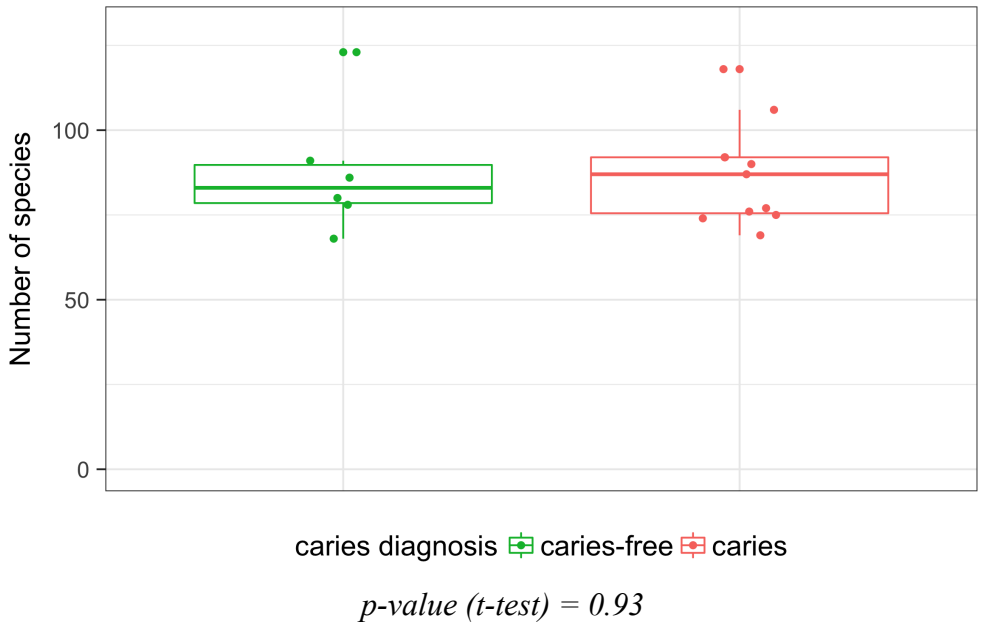
Distributed Stochastic Neighbor Embedding (t-SNE) (van der Maaten et al. 2008), and Principal Component Analysis (PCoA) (Pearson 1901) were applied. For NMDS, Bray-Curtis dissimilarity index was used. For tSNE, Euclidean distance metric and perplexity of 5 were used. For PCoA, the proportion of variability added by additional dimensions was examined through the scree plot.

Top abundant species were compared for each feature group by their rankings within the group. For the subjects with two longitudinal saliva samples, change in alpha diversity, and change in Principal Component (PC) values were studied (Whittaker 1972).

2.1. Number of genera/species

From all samples, 81 genera and 214 species, based on 360,907 gene families and 298 pathways, were identified. In comparing samples, no significant differences were noted in the number of species based on caries status (figure 4).

Figure 4. Species in each group



2.2. Abundance of bacterial species

The species-level abundance ranking of the samples was tabulated (table 2). Differential abundances were observed between caries and caries-free groups: *Actinomyces sp HPA0247* and *Actinomyces graevenitzii* demonstrated greater mean abundance in carious samples, while *Neisseria subflava* had greater mean abundance in caries-free samples.

The NMDS plot revealed no significant distinction between samples based on the presence of caries (figure 5). The tSNE plot revealed lack of clustering, likely attributable to a small sample size (figure 6). The PCoA plot showed clustering of subjects with caries at the upper center portion of the plot, with the Scree plot demonstrating that 2-3 principal components contribute to total variance (figure 7).

Table 2. Species-level abundance in salivary microbiome

	genefamily (genus level)	caries-free	caries
1	Rothia_mucilaginosa	1	1
2	Streptococcus_parasanguinis	2	2
3	Veillonella_unclassified	3	3
4	Prevotella_histicola	7	4
5	Prevotella_melaninogenica	6	5
6	Neisseria_unclassified	4	6
7	Actinomyces_graevenitzii	30	7
8	Porphyromonas_sp_oral_taxon_279	10	8
9	Actinomyces_sp_ICM47	13	9
10	Bifidobacterium_longum	9	10
11	Atopobium_parvulum	14	11
12	Rothia_dentocariosa	12	12
13	Haemophilus_parainfluenzae	5	13
14	Actinomyces_sp_HPA0247	190	14
15	Megasphaera_micronuciformis	15	15
16	Prevotella_salivae	11	16
17	Veillonella_atypica	17	17
18	Streptococcus_salivarius	16	18
19	Veillonella_dispar	28	19
20	Prevotella_pallens	19	20
21	Neisseria_flavescens	18	27
22	Neisseria_subflava	8	30
23	Alloprevotella_rava	20	34

Figure 5. NMDS plot of identified species

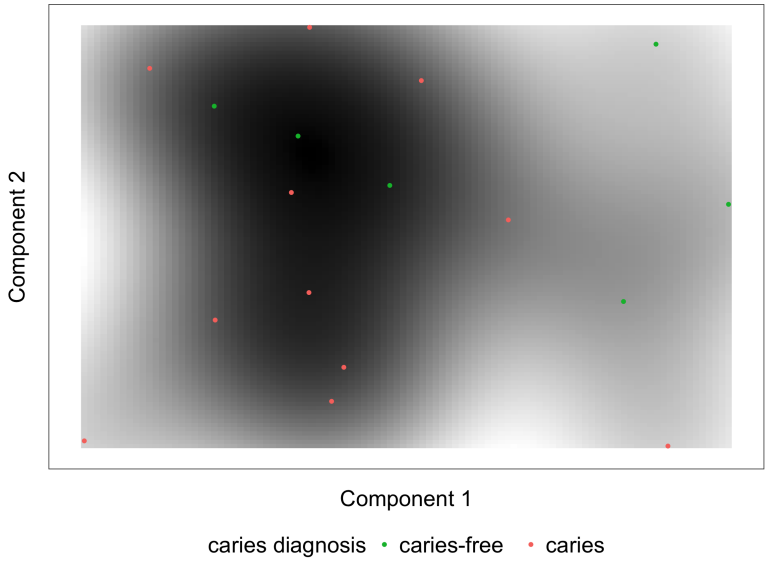


Figure 6. tSNE scatter plot

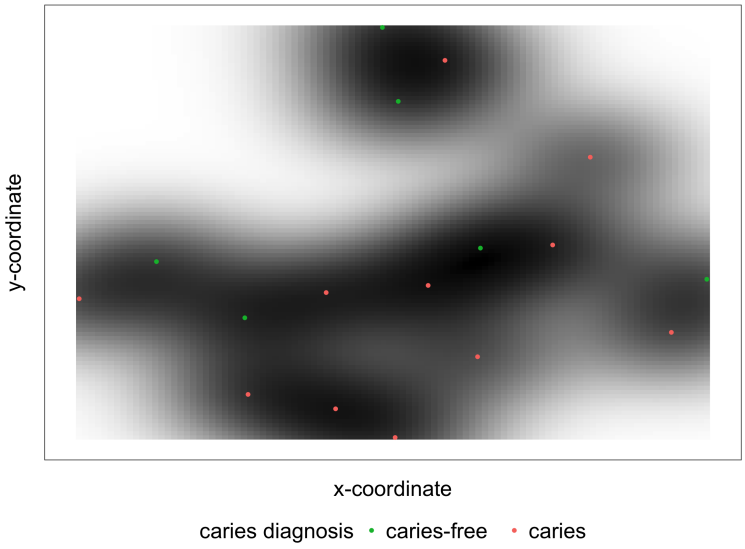
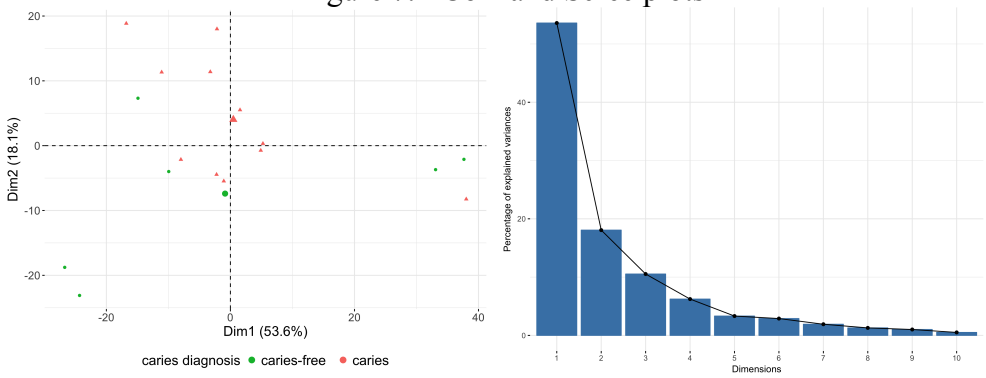


Figure 7. PCoA and Scree plots



2.3. Alpha diversity and beta diversity

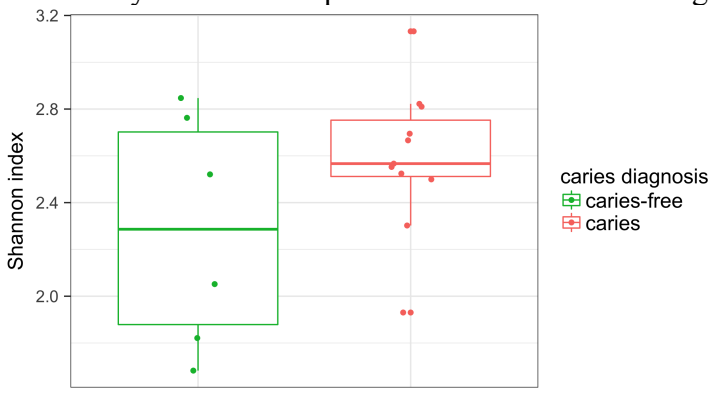
At baseline, alpha and beta diversities, at the species level, were assessed.

Alpha diversity, demonstrating species richness, through the number of taxa in the salivary microbiome, included Shannon diversity index (figure 8, figure 9), Simpson-Gini diversity index (figure 10), richness, and dominance. Richness was measured as the number of observed species within each sample given only top 50% or top 80% of the species (figure 12). Dominance represented the proportion of the abundance of the most abundant species (figure 13).

Beta diversity represented the differences of taxonomic abundance from different samples (figure 14). Beta diversity was measured using average Bray dissimilarity within each feature group.

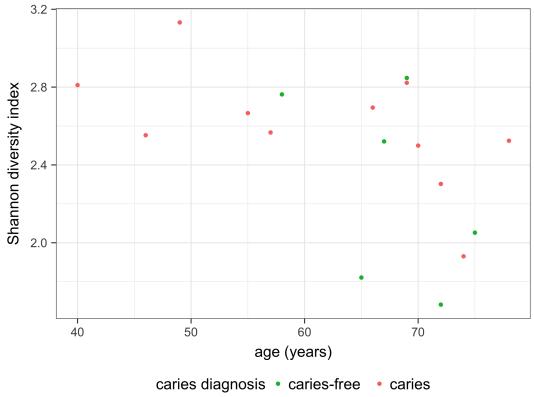
The salivary microbiome, as presented by the samples, showed a greater tendency for more diversity in caries-associated samples though there was not a significant difference in number of species between the samples. Specifically, older patients demonstrated less diversity, and with caries status, the diversity observed initially with caries is less clear (figure 9, figure 11).

Figure 8. Shannon diversity index at the species-level as mean and range in each group



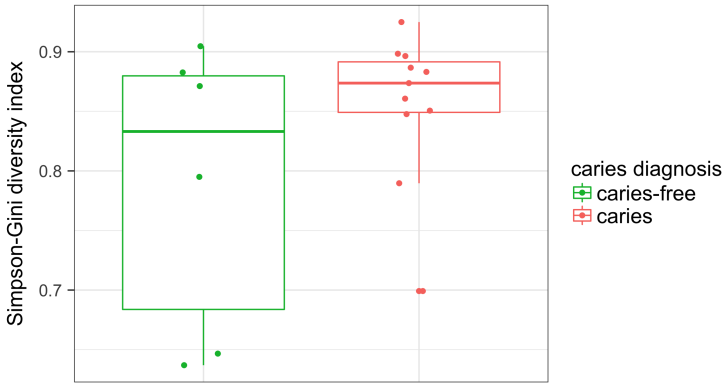
p-value (t-test) = 0.13

Figure 9. Linear model of Shannon diversity index at the species-level and age



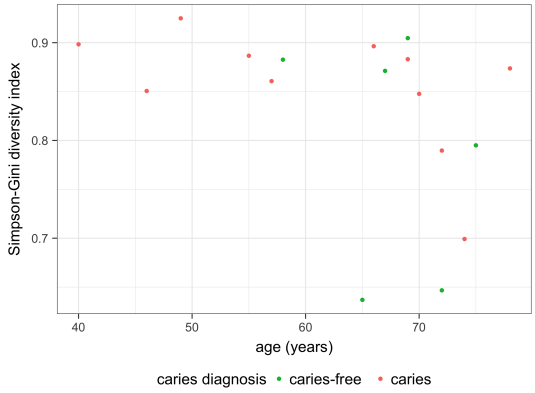
p-value = 0.28 (caries), 0.07 (age)

Figure 10. Simpson-Gini diversity index at the species-level as mean and range in each group



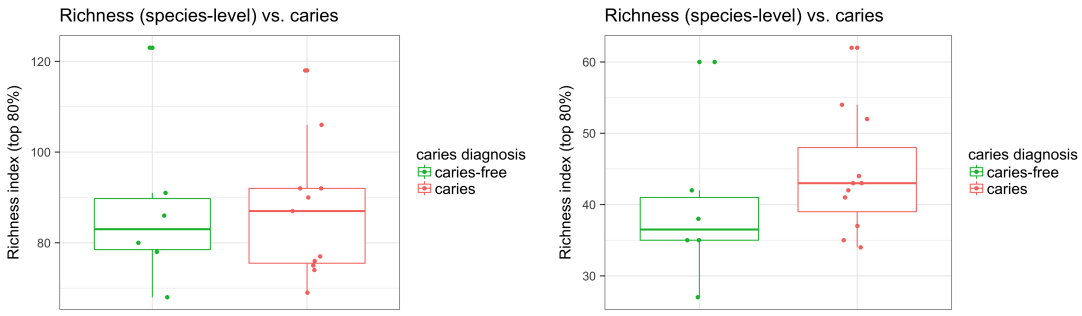
p-value (t-test) = 0.15

Figure 11. Linear model of Simpson-Gini diversity index at the species-level and age



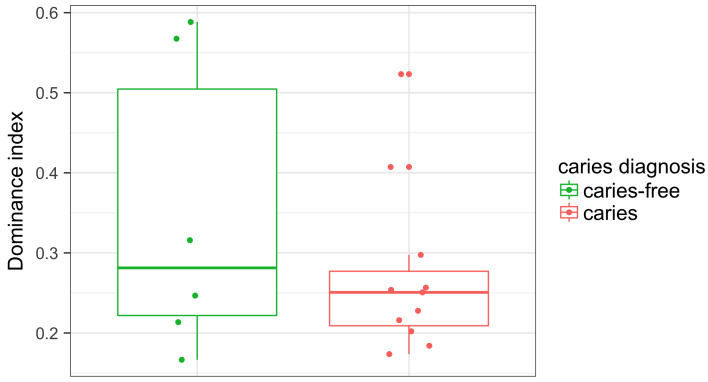
p-value = 0.28 (caries), 0.18 (age)

Figure 12. Richness index at the species-level (50%/80%)



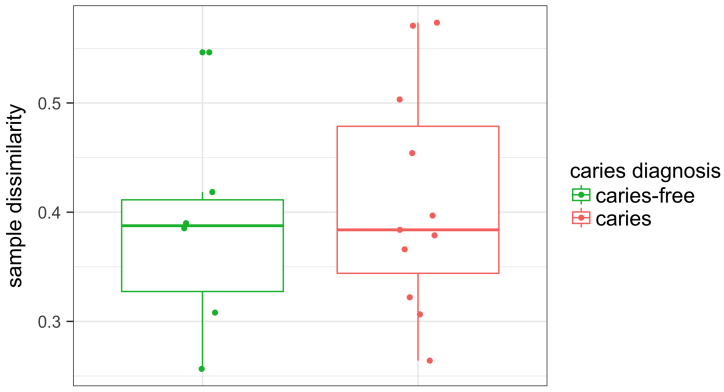
p-value (t-test) = 0.93, 0.34

Figure 13. Dominance index at the species-level according to caries status



p-value (t-test) = 0.28

Figure 14. Sample dissimilarity as measured by beta diversity at species-level



p-value (t-test) = 0.61

2.4. Longitudinal analysis

Longitudinal changes in the salivary microbiome for 4 subjects with 2 samples were evaluated for comparison (Table 3).

Between visits 1 and 2, the subjects had the following course:

Subject 002 had undergone 9 months of treatment consisting of 18 natural tooth preparations for FDP, 3 implants placements, bone graft, crown lengthening, and 6 tooth extractions. The subject also had two, 1-week courses of antibiotic prophylaxis in conjunction to treatment during that time period (one course is 500mg Amoxicillin, 3 times a day, for 7 days).

Subject 005 had undergone 9 months of treatment consisting of 7 natural tooth preparations for FDP, bone graft, and 1 tooth extraction.

Subject 007 had undergone 8 months of treatment consisting of temporary placement of a maxillary removable complete denture, 5 natural tooth preparations for FDP, 4 implant placements, 4 definitive tooth-borne FDP placement. The subject also had a single, 5-day course of antibiotic prophylaxis in conjunction to treatment during that time period (500 mg Amoxicillin, 3 times a day, for 5 days).

Subject 010 had undergone 2 months of treatment consisting of 16 natural tooth preparations.

Within and between subject analyses were graphically assessed with spaghetti plots of number of species (figure 15), alpha diversity using the Shannon-Wiener entropy index (figure 16), Simpson-Gini diversity index (figure 17), and dominance index (figure 18). The very small sample size limits our ability to make robust inferences, but we did note that diversity increased with treatment. Notably, subject 002 exhibited a different pattern compared to the other 3 subjects discussed here. Specifically, there is a noticeable change in the Simpson-Gini diversity index and the species-level dominance index specific to subject 002 between the first and second visits/samples (figure 17, figure 18).

Table 3. Demographic and clinical data of subjects with 2 samples (visits)

id	caries	perio	gingivitis	diabetes	smoker	age	gender	implants
002	0	0	1	0	1	75	m	AB
005	1	1	1	1	1	70	m	AB
007	0	0	0	0	1	65	f	A
010	1	1	1	0	0	72	m	AB

A: Implant placement during treatment, B: Implant placement prior to treatment, AB: Implant placement prior to treatment and after treatment

Figure 15. Number of species between visits and subjects

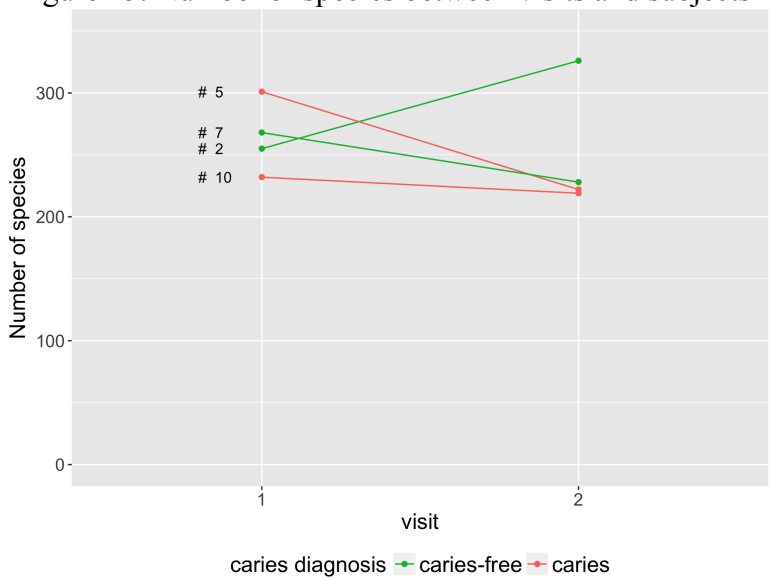


Figure 16. Shannon entropy index at the species-level between visits and subjects

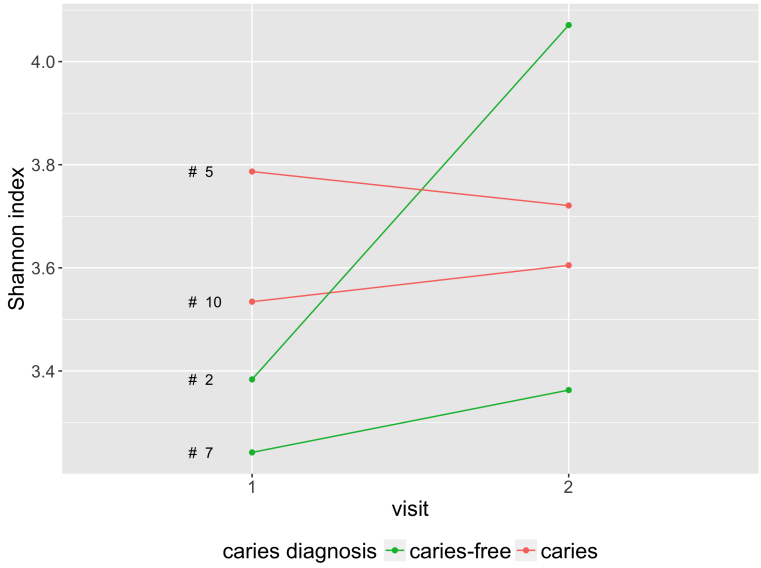


Figure 17. Simpson-Gini diversity index at the species-level between visits and subjects

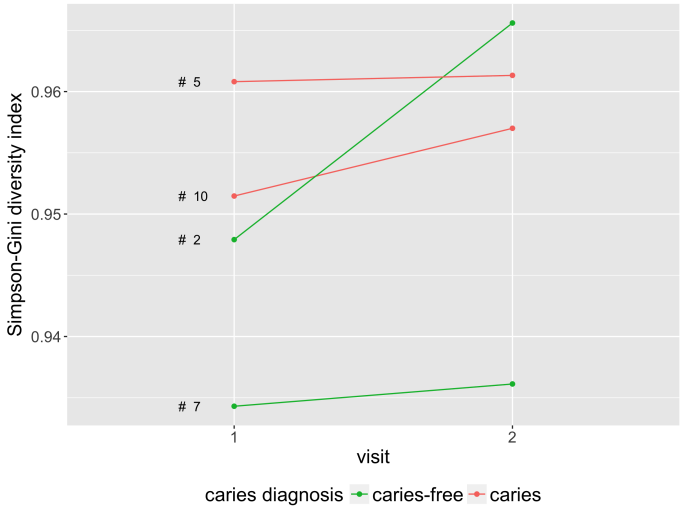


Figure 18. Dominance index at the species level between visits and subjects

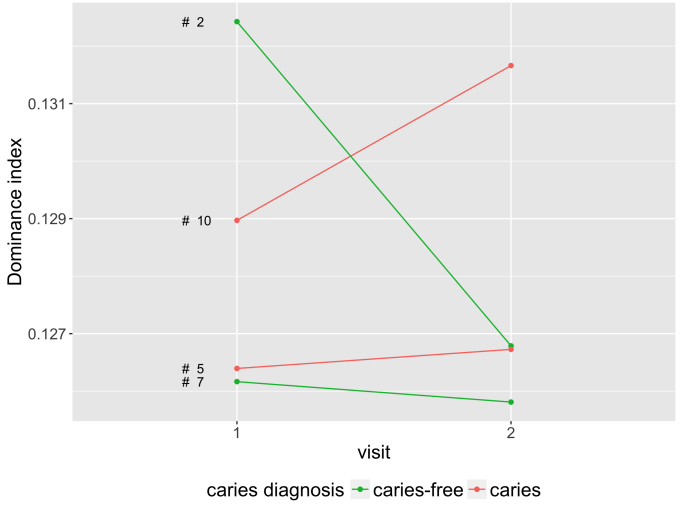
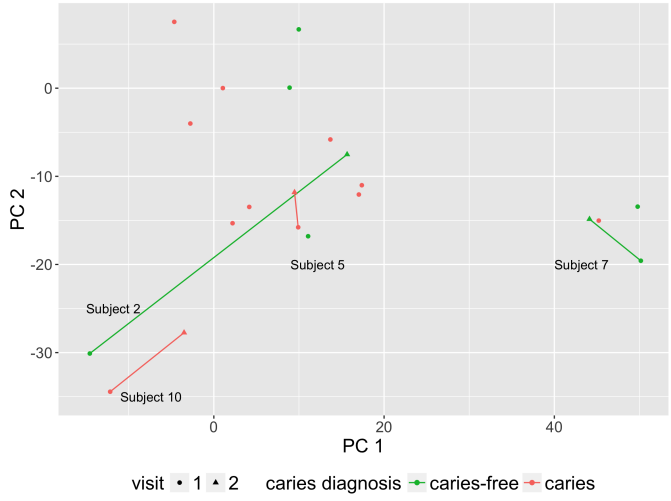


Figure 19. Change in PC between visits and subjects at the species-level



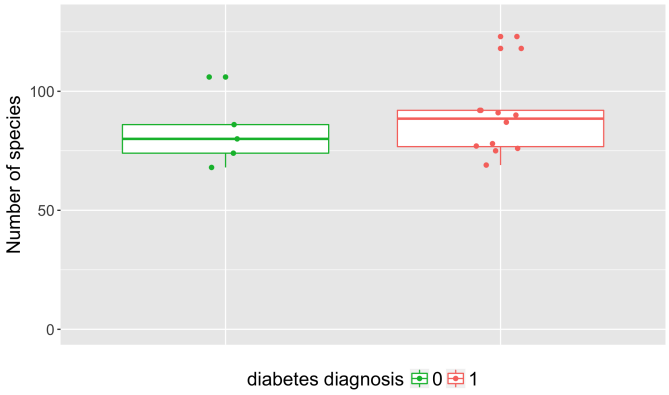
3. Salivary metagenome analysis: Variables

Diversity indices were compared across clinical characteristics and other medical information or comorbidities such as dental caries diagnosis, gingivitis, periodontal disease history, diabetes, gender, and smoking status. Findings related to diabetes and periodontal disease history were notable.

3.1. Diabetes analysis

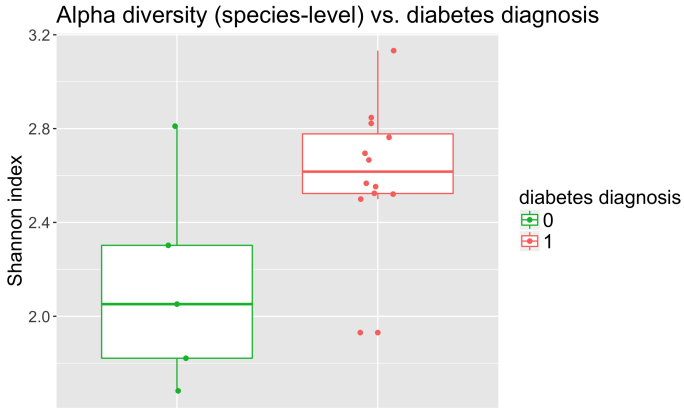
We found that the salivary microbiome of patients with diabetes was more diverse compared to those without diabetes. Though the number of species did not show any important difference (figure 20), alpha and beta diversity indices were different between the groups (figures 21-24). The NMDS, tSNE, and PCoA plots revealed a distinct clustering of samples associated with diabetes (figures 26-28).

Figure 20. Species in each group (diabetes)



p-value (t-test) = 0.93

Figure 21. Shannon diversity index at the species-level as mean and range in each group (diabetes)

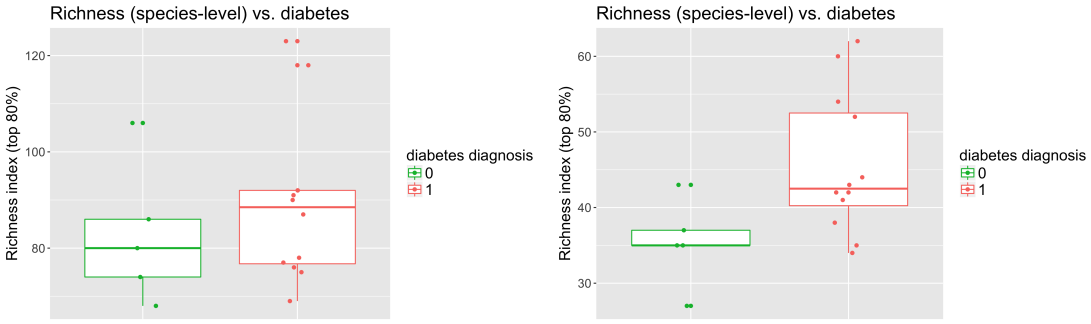


p-value (t-test) = 0.015

Figure 22. Simpson-Gini diversity index at the species-level as mean and range in each group (diabetes)

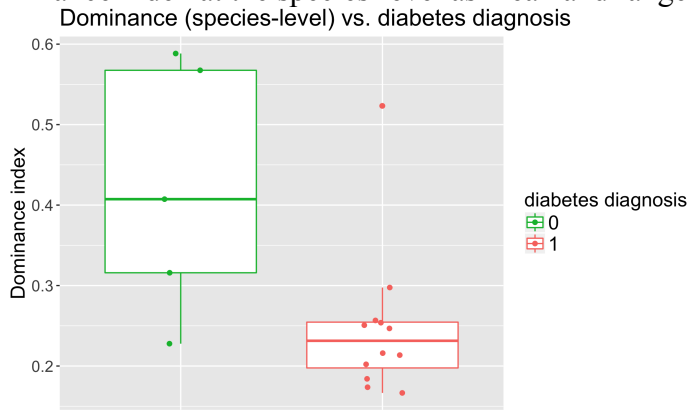
p-value (t-test) = 0.013

Figure 23. Richness index at species-level (50%/80%) (diabetes)



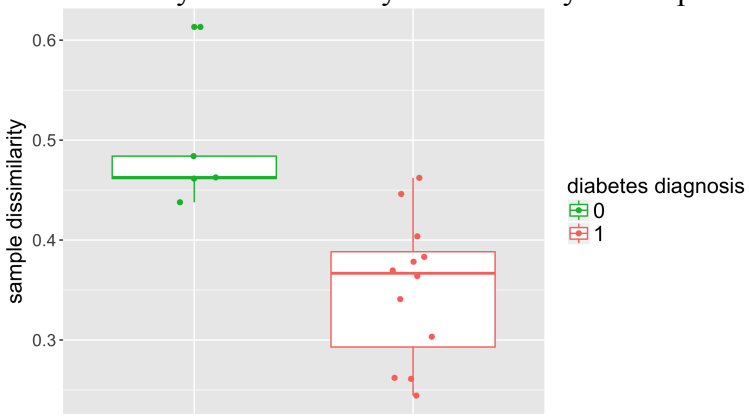
p-value (t-test) = 0.48, 0.04

Figure 24. Dominance index at the species-level as mean and range in each group



p-value (t-test) = 0.013

Figure 25. Sample dissimilarity as measured by beta diversity at the species-level (diabetes)



p-value (t-test) = 0.002

Figure 26. NMDS plot of identified species (diabetes)

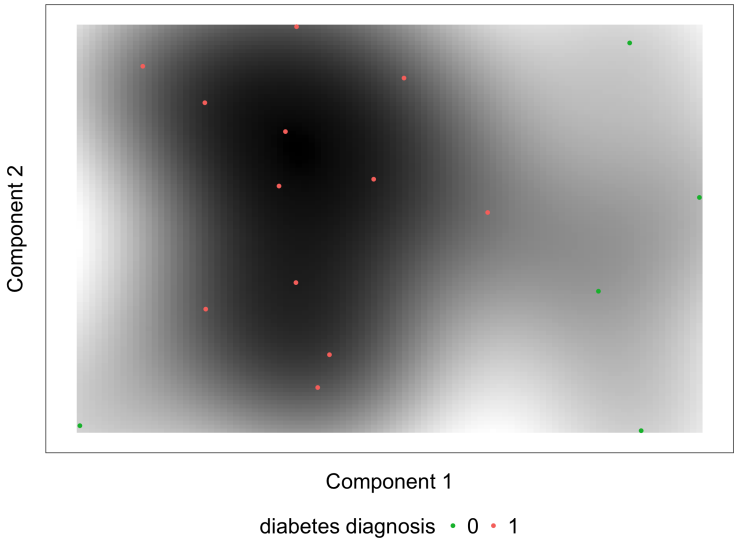


Figure 27. tSNE scatter plot (diabetes)

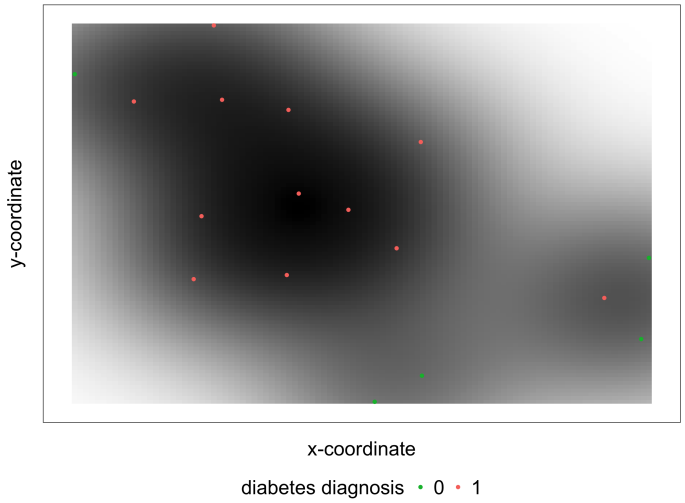
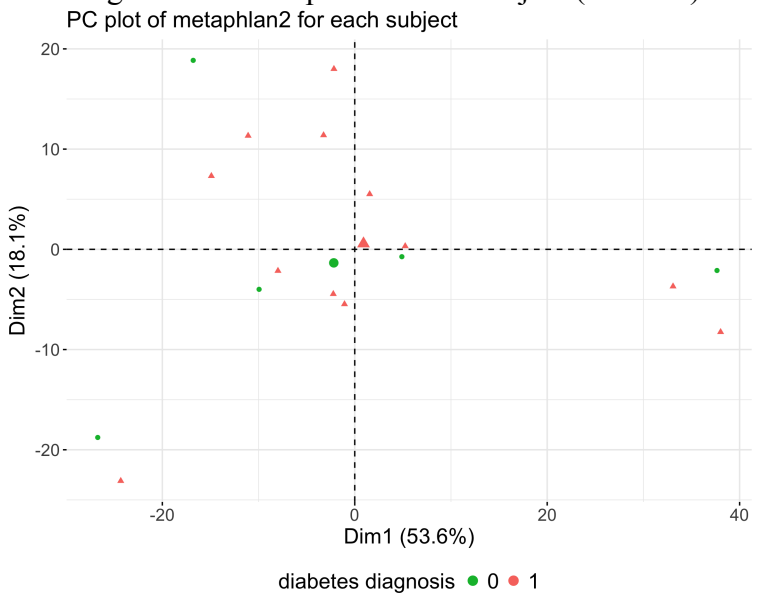


Figure 28. PCoA plot for each subject (diabetes)



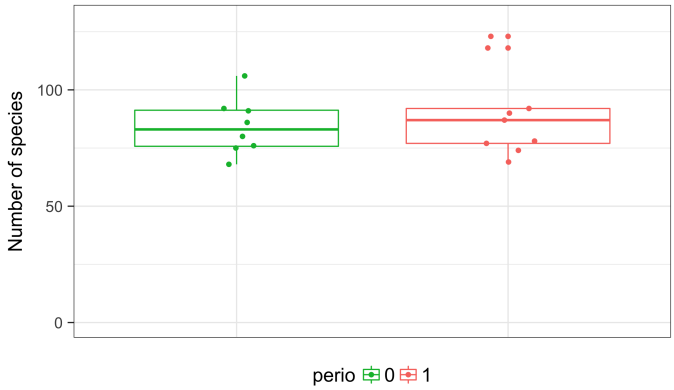
3.2. Periodontal disease history analysis

The salivary microbiome of patients with a previous history of periodontal disease was more diverse compared to those without history of periodontal disease. However, beta diversity analysis between groups of patients who had a history of disease versus no disease revealed the opposite (figure 34), signifying that diversity was similar between samples derived from previous history of periodontal disease than that of samples without a history of disease. The

number of species did not show any important difference (figure 29), whereas alpha and beta diversity indices differed between the groups (figures 30-34).

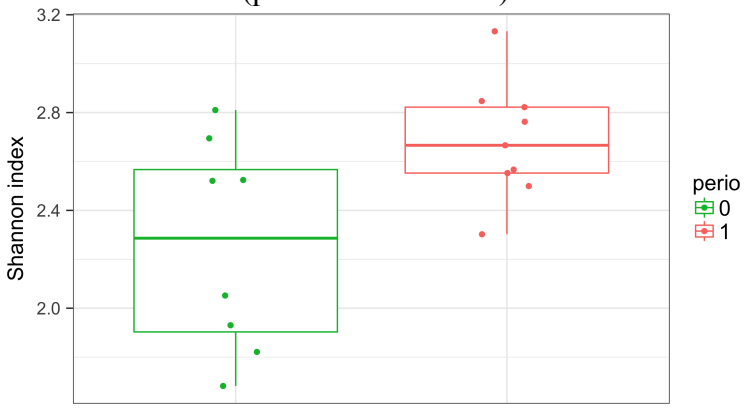
The PCoA plot (figure 37) revealed a distinct clustering of samples associated with disease, but NMDS and tSNE, did not reveal any notable patterns (figure 35, figure 36).

Figure 29. Species in each group (periodontal disease)



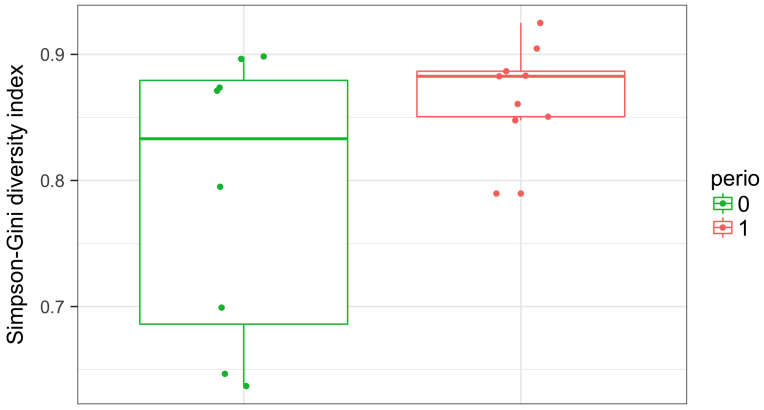
p-value (t-test) = 0.49

Figure 30. Shannon diversity index at the species-level as mean and range in each group (periodontal disease)



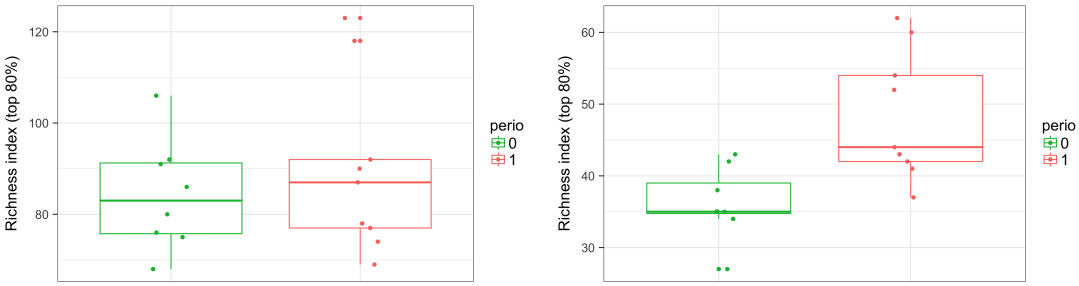
p-value (t-test) = 0.021

Figure 31. Simpson-Gini diversity index at the species-level as mean and range in each group (periodontal disease)



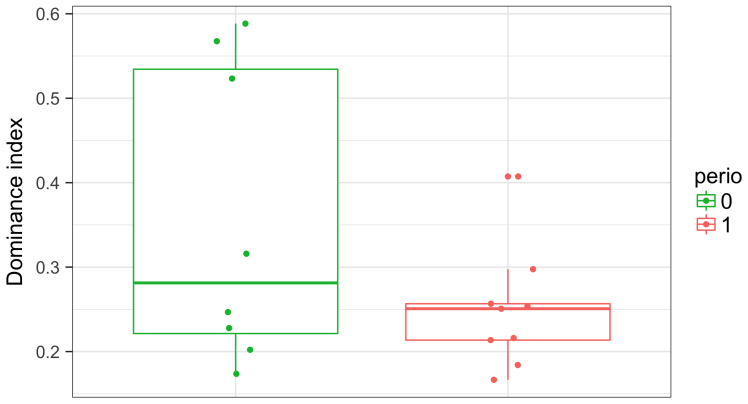
p-value (t-test) = 0.06

Figure 32. Richness index at the species-level (50%/80%) (periodontal disease)



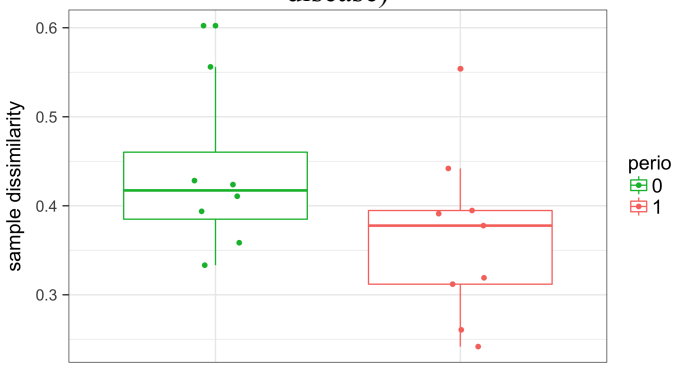
p-value (t-test) = 0.49, 0.004

Figure 33. Dominance index at the species-level as mean and range in each group (periodontal disease)



p-value (t-test) = 0.011

Figure 34. Sample dissimilarity as measured by beta diversity at species-level (periodontal disease)



p-value (t-test) = 0.14

Figure 35. NMDS plot of identified species (periodontal disease)

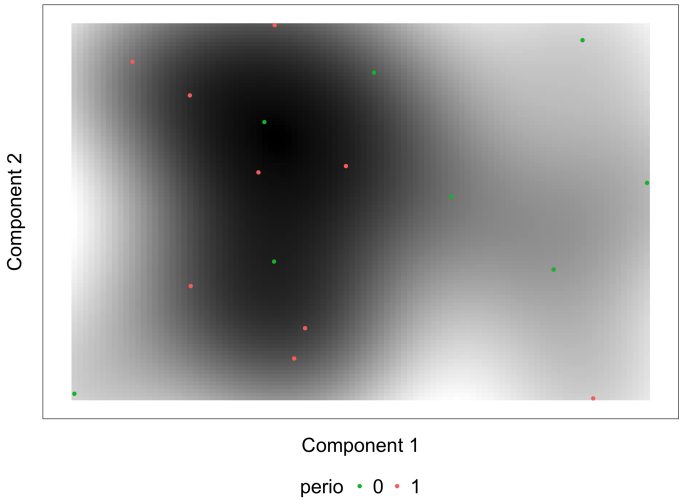


Figure 36. tSNE scatter plot (periodontal disease)

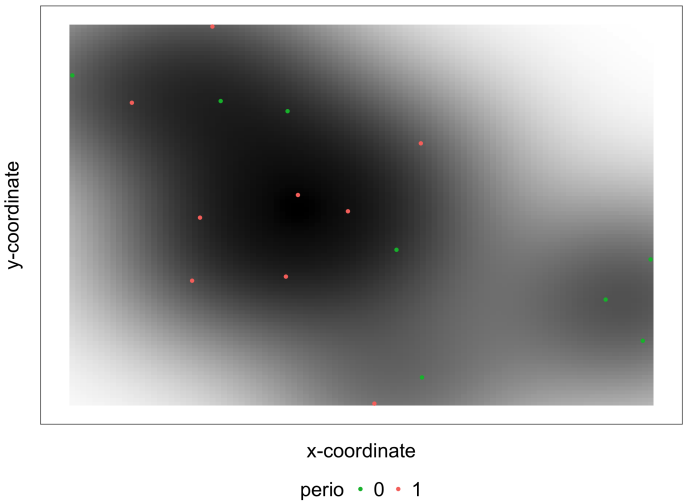
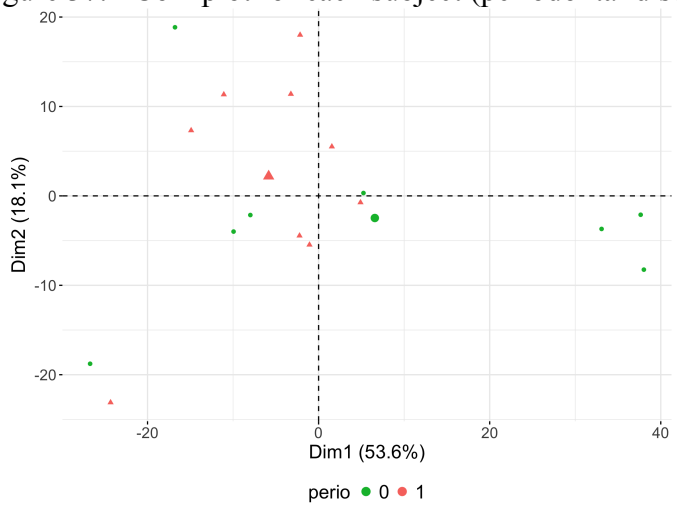


Figure 37. PCoA plot for each subject (periodontal disease)



DISCUSSION

Full mouth rehabilitation treatment is geared towards achieving health. But, thus far, oral health has been assessed using clinical parameters that indicate preservation of tissues and structures without the presence of loss or destruction due to disease and/or parafunction. A dental provider evaluates objective criteria that measure for health versus disease in a subjective manner. So even though clinical health can be readily assessed, little research has been conducted in understanding how prosthodontic treatment may impact the oral microbial status within the contemporary understanding of oral health and disease as complex, host-biofilm dysbiotic states. In an effort to improve the state of oral health, understanding how these types of restorations may affect the foundation, one's oral microbiome, may impart some insight on the efficaciousness of this approach in treatment.

This study collected unstimulated, whole saliva samples. The choices regarding the type of saliva and method of collection were based on the sampling kit utilized for the study. However, in understanding the quality of gathered information, it is important to note that different studies have had varying findings in comparing the sensitivity and comparability of bacteria detected within stimulated versus unstimulated saliva(Schafer et al. 2014; Simon-Soro et al. 2013; Yakob et al. 2014). When using culture-based methods, stimulated saliva produced better detection of specific oral bacteria(Asikainen et al. 1991; Dasanayake et al. 1995), while pyrosequencing found stimulated saliva to be more diluted(Simon-Soro et al. 2013). A recent study systematically compared the salivary microbiota of within-subject samples obtained in both stimulated and unstimulated forms, using Human Oral Microbe Identification using Next

Generation Sequencing (HOMI*NGS*)(Belstrom et al. 2016a). It determined that no significant differences in composition and proportions of the respective bacterial profiles at the species and genus level probe targets(Belstrom et al. 2016a).

This study's salivary metagenomics analysis approach based on whole genome sequencing (WGS) shotgun allowed us to obtain a more comprehensive view of the oral microbiome. WGS can reveal more unidentified and unassigned, non-human taxonomic sequences of the salivary metagenome (Lazarevic et al. 2012); it can, thereby contribute to more information regarding the actual functional potential of the microbial cells through assignments of sequences from databases organized by functional sequences(Gerlach and Stoye 2011; Meyer et al. 2008).

Previous studies have shown that salivary bacterial profiles differ between disease and non-disease statuses (Belstrom et al. 2017b; Chen et al. 2018). Differences in the abundance of particular species, namely of the genera of *Neisseria, Haemophilus,* and *Fusobacterium,* were evident when comparing compositions of microbiomes that had no active disease but a history of previous disease to those without any history of disease (Belstrom et al. 2017b). Bacterial diversity was found to be significantly higher in individuals with low caries experience than those with 10 times higher caries experience (Belstrom et al. 2017b). Similar findings have been observed for periodontal disease, in which bacteria that have known associations to disease continue to have proportionally large abundance and representation in diseased states(Ai et al. 2017; Belstrom et al. 2015).

Age, diabetes, and periodontal status appeared to also be associated with differences in the salivary microbiome in this study. Age has been found to affect the microbiome's composition, though age in of itself may not necessarily cause change but rather an individual's

health condition and associated co-morbidities such as increased presence of inflammatory markers that occur in older populations (Costalonga and Herzberg 2014; Lira-Junior et al. 2018; Nassar et al. 2014; Takeshita et al. 2016; Xu et al. 2015). Diabetes has not consistently been shown to alter the oral microbiome, though observed changes have been associated with co-morbidities such as obesity. (Goodson et al. 2017; Janem et al. 2017; Kampoo et al. 2014).

When evaluating changes incurred after therapy to treat a disease and restore health, studies have found that clinically determined improvements in oral conditions cannot be necessarily defined or quantified by bacterial abundance (Schwarzberg et al. 2014). Specifically, the notion of microbiologically diagnosing health based upon proportions of particular microorganisms associated with health and disease is not generalizable. Rather, compositions of microbial cells that promote symbiosis are unique to an individual's specific microbiome and its functions and interactions (Schwarzberg et al. 2014). In periodontal disease therapy, diseased and previously-diseased oral environments were diverse and individualized, while microbiomes without a history of disease were homogenous(Chen et al. 2018).

Longstanding treatment outcomes are ultimately not determined by prosthetic restoration, but by the ability to manage the microbial balance that is supportive towards health (Yanase and Le 2014). Management of an individual's oral microbiome through behavioral and environmental modifications may, in fact, be the key for effective prevention and treatment (Gomez et al. 2017). The insertion of dental biomaterials, like crowns and implants, within the oral cavity also impacts the microbial community's interactions. Each type of dental prosthesis has its own requirements for clinical application and laboratory fabrication (Rosenstiel et al. 2016). The FDP, in particular, has an array of material types and an array of designs that have specific parameters for placement on a tooth (Goodacre et al. 2001). Furthermore, when the

foundational tooth is compromised, be it structural deficiencies, presence of disease on hard and/or soft tissues, or previous restorations (direct restorations like amalgam and composite resin restoration, root canal therapy, posts or pins), rendering treatment with FDP is more complex (Rosenstiel et al. 2016; Zitzmann et al. 2010). These restorations create new habitats by nature of the type, composition, and structure of the prosthesis (Lin 2017; Øilo and Bakken 2015). These habitats are being formed within an already established habitat of the tooth and gingival tissues, and thus the niches that microorganisms had previously had can potentially be altered.

This present study has several limitations. Because it does not involve direct intervention, the prosthodontics treatment is non-standardized and dental providers' sequence of care is left to their discretion. Therefore, timing between sample collection varied while some patients may had received definitive restorations and provisional restorations versus only provisional restorations. Another source of heterogeneity is the large number of covariates and comorbidities involved (e.g., age, systemic health, behaviors, medications, to name a few). The basic inclusion factor being 6 fixed, tooth-borne dental prosthesis enabled various types of treatment to be accepted within the study design, including implant therapy and removable dental prostheses. While these factors introduce some variance, they also reflect 'real life' in the sense that dental treatment is rarely standardized and individual patients present with their own set of individual variation and comorbidities. Another major limitation is obviously the small sample size; this reduces the statistical power and the study's potential to make robust inferences and detect associations. Also, due to pilot and exploratory nature of the study, the findings may not be generalizable. However, the study protocol intends to recruit 30 participants (90 total samples), enhancing the study's ability to make formal within- and between-subjects comparisons and inferences.

Through the study of the oral microbiome, insight and improvements in preventive and interventional clinical practices can be made. Manipulation of the oral biofilm and its interactions may lead to more effective treatments. Likewise, distinguishing how the oral microbiome is altered and reacts in different states of health and disease may support the establishment of preventive measures such that disease can be prevented or minimized. The developing findings have led to the growth of the field of microbiomics – the therapeutic translation of microbiotic information to personalized health care (Glurich et al. 2015; Scannapieco 2013; Zarco et al. 2012).

Caries prevention is being investigated through active and passive means of immunization. Both approaches entail the targeting of specific gateway pathogens like MS. For active immunization, particular components involved in interactions such as bacterial adhesion, glucan development or binding, and cell wall synthesis, can serve as immunogenic targets to illicit antibody formation and delivered through vaccination (Costalonga and Herzberg 2014). The passive means of acquiring immunity have been explored with antibody administration through routes like dietary supplements and dental trays.

In spite of the significant strides towards understanding the oral cavity and its microbiomes, limitations persist. Most of the data available pertain to bacterial species and do not characterize or delve into other facets of the oral environment such as fungal species (mycobiome) and viruses (viriomes) (Zaura et al. 2014). Additionally, profiling the genomic composition of a microbiome does not fully explain the functions provided by the microorganisms found within it. Meta-transcriptomics, proteomics, and metabolomics can further explain the process by which symbiosis is maintained or dysbiosis persists, by examination of the biological functions and metabolic activity of the microbial communities

(Bao et al. 2015; Belstrom et al. 2017a; Duran-Pinedo et al. 2014; Duran-Pinedo and Frias-Lopez 2015; Fabian et al. 2008; Xu and Gunsolley 2014).

CONCLUSION

The impact of the oral cavity and its health is important to general health and quality of life. With the progress of novel sequencing technologies and techniques, insights into the oral cavity and its constituents can be gained. This more expansive knowledge set can provide significant means for understanding the biological basis of health and disease of the oral environment. This is especially critical for dental providers who are invested in restoring the dentition and promotion oral health, as common diseases of caries and periodontitis continue to pose clinical problems and compromise restorations. Through this exploratory study, a pattern of change during prosthodontic treatment was evident, although the intricacies of what and why the salivary microbiome changes have yet to be fully assessed. The ultimate findings, however, can contribute to the growing field of microbiomics in which intentional manipulation of microorganisms and their functions and activities may be applied to develop and render more effective treatments.

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